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Future Therapeutic Strategies: Implications for Brk Targeting

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1. Introduction

Over the last two decades the survival of patients with breast cancer has improved significantly. This is partly as a result of national screening programmes resulting in earlier detection, but also due to major advances in the range of therapies that are now available for patients. However, spread of the disease and resistance to therapy is still an issue for many patients. It is therefore vitally important that the current rate of treatment advances continues for the foreseeable future. In addition to understanding resistance, and therefore generating solutions to overcome it, there is a need for new drug targets to be identified. This chapter will review the published work that has lead to Brk being identified as a potential new target for breast cancer therapy, and discuss the practicalities and implications of a Brk-targeted therapy.

2. Brk discovery and identification

The intracellular protein kinase, Brk (known as **br**east tumour **k**inase, or protein tyrosine kinase 6, PTK6), has been implicated in the development and progression of a number of different tumor types. It was first identified in 3 separate studies in the early 1990s. Initially it was identified in a study to determine which tyrosine kinases were expressed in human melanocytes (S.-T. Lee et al., 1993). Publication of the full-length sequence followed in 1994 after it was cloned from metastatic breast cancer as part of a screen to identify novel kinases with therapeutic potential, i.e. those that were expressed in breast cancers but were not found in normal mammary tissue (Mitchell et al., 1994). Simultaneous identification of the murine orthologue, sik (Src-related intestinal kinase), in mouse intestinal cells was achieved through the generation of a library of kinase catalytic domains (Siyanova et al., 1994).

3. Structure of gene and protein

3.1 *PTK6* gene

The *ptk6* gene comprises 8 exons which span 10kb (Mitchell et al., 1997) and encodes a protein of 451 amino acids in size (Mitchell et al., 1994). Only the boundary between Exons 1 and 2 is conserved with members of the src family (H. Lee et al., 1998; Mitchell et al., 1997) whereas the gene structure of other src family members exhibits high levels of evolutionary conservation, suggesting that *ptk6* belongs to a related, but distinct family of tyrosine

kinases. As 6 out of 7 exon boundaries however, are conserved with *Dsrc41*, *ptk6* may share a common ancestoral evolution with *Dsrc41* (Mitchell et al., 1997).
Fluorescent in situ hybridization studies assigned the *ptk6* gene to chromosome 20q 13.3 and initial analysis of the promoter region identified a number of cis-acting elements including those for Sp1, SIE, AP2 and NFκB (Mitchell et al., 1997). Both NFκB and Sp1 have been shown to bind to cis-acting elements within the promoter region, suggesting that they can play a role in regulating *ptk6* gene transcription (Kang et al., 2002).
Crompton and colleagues reported that the *ptk6* gene sequences derived from normal and tumour tissue were identical, suggesting that the ability of Brk/PTK6 to regulate cancer cell growth was not associated with gene mutations (Mitchell et al., 1994). A search of the COSMIC database in March 2011 (Catalogue of Somatic Mutations in Cancer, hosted by the Wellcome Trust Sanger Institute) revealed that out of 359 tumour samples analysed, only 1 contained a mutation, which provides further corroboration that Brk's role in tumour development occurs as a result of aberrant expression and/or altered cellular localization (see section 5).
In addition, an alternatively spliced variant of Brk has been identified that codes for a 134 amino acid protein, termed λ5 (Mitchell et al., 1997) or ALT-PTK6 (Brauer et al., 2011).

3.2 Brk/PTK6 protein

The protein product of the *PTK6* gene is the non-receptor tyrosine kinase, Brk. It comprises SH3, SH2 and kinase domains and shares 45% amino acid sequence homology with DSRC41 and 44% with human Src (H. Lee et al., 1998; Mitchell et al., 1997). As a 451 amino acid protein, Brk has a predicted molecular weight of 52kDa but typically resolves to around 48kDa on an SDS-PAGE gel. The protein also comprises an SH2-kinase linker region and a C-terminal tyrosine residue (Y447), both of which are involved in regulation of catalytic activity (Figure 1).
The backbone dynamics and solution structure of the SH2 domain of Brk were proposed by Yonsei University in 2004 (Hong et al., 2004). The peptide used in these studies had a dissociation constant of around 60uM. This is a much weaker Kd than had previously been reported for Src family members; again highlighting that although similar to Src, Brk belongs to a distinct family. Differences in Kd values are indicative of different interactions between 'receptors' and their ligands. In support of this, BrkSH2-ligand interactions differ from those involving canonical SH2 domains suggesting that Brk's SH2 domain might have unique binding features that are required for its specific ligand interactions. In addition, NMR data have suggested that the SH3 domain of Brk undergoes severe conformational instability in response to a change in the pH of its environment (Koo et al., 2002). Changes in conformation as a result of subtle changes in cellular pH could therefore alter possible Brk-substrate interactions. In indentifying the intramolecular SH3 binding site, Qiu and Miller were also able to demonstrate the importance of the SH3 domain in regulating Brk-substrate interactions (Qiu and Miller, 2004).
In contrast to src family members, where the interaction between the SH2-kinase linker region and the kinase domain inhibits the enzyme's kinase activity, the linker-kinase interaction is fundamental for Brk's catalytic activity (Kim and S.-T. Lee, 2005). Conversely, linker-SH3 domain interactions negatively regulate the kinase. Proline residues 175, 177 and 179 in the N-terminal part of the linker are required for the linker-SH3 domain interaction, which, alongside the C-terminal phospho-tyrosine-SH2 interaction hold the protein in a negative conformation (Kim et al., 2007).

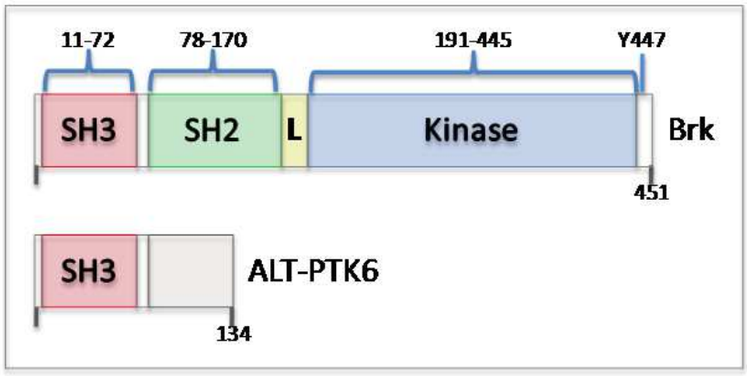

Fig. 1. Schematic representation of Brk and its alternative isoform ALT-PTK6. PTK6 consists of SH3 (red), SH2 (green) and kinase (blue) domains. The linker region (L) containing prolines 175, 177 and 179 is in yellow.

The alternatively spliced isoform, ALT-PTK6, is 15kDa in size and comprises the SH3 domain and a novel proline rich sequence but is truncated before the end of the SH2 domain so lacks functional SH2 and kinase domains (Mitchell et al., 1997). The biological role of this isoform of Brk is unknown, but it is possible that it competes with wild-type Brk for SH3 binding potentially acting as a competitive inhibitor (Brauer et al., 2011).

4. Brk expression profile

4.1 In normal cells

Physiological Brk expression is typically found within a number of normal epithelial tissue types where it is involved in differentiation and has a negative role in cell proliferation. In normal tissue Brk/sik expression is restricted to the cell layers immediately above the proliferative cell zone in these epithelia (Vasioukhin et al., 1995).

Brk/sik is highly expressed in the gastrointestinal (GI) tract where it is present within the non-dividing villus epithelium of the small intestine as well as detectable in the crypt cells post-irradiation (Llor et al., 1999; Vasioukhin et al., 1995; Haegebarth et al., 2009). In the colon Brk/sik was expressed at high levels in the upper crypts in cells undergoing terminal differentiation (Llor et al., 1999), and Brk expression has also been detected in the nuclei of normal luminal prostate epithelial cells (Derry et al., 2003) and oral epithelia (Petro et al., 2004).

Within the epidermis of the skin Brk expression was detected mainly in differentiating layers in the suprabasal keratinocytes (T. C. Wang et al., 2005). *In vitro* studies have supported a role for Brk/Sik in calcium-induced keratinocye differentiation, which was accompanied by the elevation of the epidermal differentiation markers such as Keratin10 or Filaggrin (Tupper et al., 2011; T.C. Wang et al., 2005; Vasioukhin and Tyner, 1997).

Perhaps most surprisingly, although Brk is over-expressed in breast tumours, it is not detected in normal mammary tissues or fibroadenomas (Barker et al., 1997), or at various stages of mouse mammary development (Llor et al., 1999).

4.2 In breast cancers

Brk is known to be low or undetectable in normal mammary tissue and benign lesions, but, in contrast, has been shown to be highly detectable in breast tumours. Typically, Brk was

detected in approximately two thirds of the breast tumours analysed, where approximately a third of these are overexpressed by levels ranging from five-fold to forty three-fold compared to normal tissue (Barker et al., 1997).
Brk is highly expressed in lobular and medullary carcinoma samples (Lukong et al., 2005), and its expression has been reported in up to 86% of breast cancers (Aubele et al., 2007; Ostrander et al., 2007; Harvey et al., 2009). In patient samples Brk mRNA expression correlated with an increase in histological tumour grade (Harvey et al., 2009) and immuno-staining revealed an increased level of Brk protein in higher grade tumours (Chakraborty et al., 2008) and specimens with a higher percentage of carcinoma within the sample (Ostrander et al., 2007).

4.3 In other cancers

Brk expression has been detected in a number of cancer types from a variety of tissues. It was found to be highly expressed in 70% of high-grade, serous ovarian carcinomas, but absent in normal ovarian surface epithelia. Expression of Brk was also detected in approximately half of the ovarian cancer cell lines examined, but was again undetected in immortalized ovarian surface epithelium (Schmandt et al., 2006).
Although Brk expression has been detected in the normal human GI tract, it is highly expressed in colon tumour samples and cell lines (Derry et al., 2000; Llor et al., 1999). Indeed, Brk mRNA expression has also been evaluated and detected in tissue in origin from normal colon, polyps and tumours (Chen W et al., 1999).
Brk is expressed in secretory epithelial cells in prostate adenocarcinoma where localisation is believed to be important, as nuclear Brk has been correlated with lower tumour grade (Derry et al., 2003). There were detectable levels of Brk in human oral squamous cell carcinomas (Petro et al., 2004), head and neck squamous cell carcinoma (HNSCC) specimens (Lin et al., 2004), as well as in a large proportion of cutaneous T-cell lymphomas and other transformed T- and B-cell populations (Kasprzycka et al., 2006).

4.4 Regulation of expression

Although the *ptk6* gene promoter has been analysed, and Sp1 and NFkB are proposed to regulate gene transcription (Kang et al., 2002), little is known about the cellular events that bring about regulation of *ptk6* gene transcription in breast cancers. Brk expression is not cell cycle dependent (Barker et al., 1997), nor have there been any studies to suggest an increase in Brk in response to exogenous or autocrine factors known to be involved in breast cancer progression such as oestrogen, progesterone and ErbB receptor ligands. *ptk6* expression may occur as a result of Klf9 transcription factor activity, although it is likely that *ptk6* expression is an indirect effect of Klf9 action (Simmen et al., 2007).
ptk6 is co-expressed with *ErbB2* in breast cancers (Born et al., 2005) and amplification of the *ptk6* gene alongside E*rbB2* gene amplification has been reported (Xiang et al., 2008), however this latter observation does not appear to be consistent with studies on other tumour cohorts (Irie et al., 2010).
Regions of chromosome 20q are frequently amplified in breast cancer (Isola et al., 1995; Kallioniemi et al., 1994) so it is possible that over expression of the PTK6 gene occurs as a result of this amplification event. However, this is unlikely to be the complete picture. Aubele and colleagues reported that *ptk6* over expression in breast cancer is unlikely to be attributed solely to gene amplification (Aubele et al., 2009). In their study, the *ptk6* gene was

amplified in only 15% of 389 Brk-positive tumours and a further 30% of tumours had polysomy of chromsome 20. A normal gene copy number was detected in 55% of invasive breast cancers. These data are supported by reports showing that 85% breast cancers express Brk (Harvey et al., 2009) and between 60 and 86% of breast cancers have elevated Brk protein compared to normal breast tissue (Aubele et al., 2007; Barker et al., 1997; Ostrander et al., 2007).

Taken together these data show that, while in some breast cancers *ptk6* over-expression is likely to be related to amplification events, elevated Brk protein also arises as a result of alternative mechanisms. As Brk has not been detected in normal human mammary epithelial cells, or during development of the mouse mammary gland (Llor et al., 1999), the events triggering expression (irrespective of whether the gene is amplified or not) still remain elusive.

5. Brk localization

Brk has been reported to have different functions in different tissue types; for example, in normal tissues Brk's role appears to be related to regulating the differentiation process, whereas in tumour cells Brk promotes proliferation and cell survival. Variations in cellular localisation are thought to be one of the underlying factors contributing to Brk's opposing roles in differentiation and proliferation. Alterations in cellular localization will no doubt affect the variety of substrates and binding partners that are available for association with Brk, thereby contributing to the different functions and effects that have been ascribed to expression of the *ptk6* gene (reviewed in Brauer and Tyner 2010).

Myristoylation is a post- or co-translational protein modification, whereby a fatty acid-derived group is attached to an N-terminal amino acid. Such modifications are important as they allow proteins to associate directly with membrane structures rather than relying on interactions with additional membrane-associated proteins for membrane localisation (reviewed in Sorek et al., 2011). Although Brk is structurally related to Src, it lacks the amino-terminal myristoylation site. Without this myristoylation site, Brk is not able to interact directly with the plasma membrane, and as it lacks a nuclear localization sequence (NLS), Brk was originally thought to be solely a cytoplasmic kinase (Mitchell et al., 1994).

Without cellular targeting via an NLS or myristoylation, Brk's cellular localization is not tightly regulated; as a result, we now know that it can be found localized in different cellular compartments based on its protein-protein interactions. It has been reported at the membrane via association with ErbB growth factor receptors (Aubele et al., 2010) and the adamalysin ADAM15 (Zhong et al., 2008), in the cytoplasm interacting with paxillin and mitogen activated protein (MAP) kinase (Aubele et al., 2008; Chen et al., 2004) as well as in the nucleus through interactions with RNA binding proteins such as Src-associated in mitosis-68 (Sam68) and the Sam68-like mammalian proteins, SLM1 and SLM2 (Derry et al., 2000; Haegebarth et al., 2004). In normal human prostate epithelial cells and well-differentiated prostate carcinomas, Brk was localized in the nucleus whereas poorly differentiated prostate tumours had cytoplasmic Brk (Derry et al., 2003). In oral epithelia Brk was localized in the nucleus and cytoplasm, but within the perinuclear regions in the oral squamous carcinoma cells (Petro et al., 2004). These data suggest that Brk's cellular localisation may affect its role in oncogenesis as much, if not more, than the level of over-expression. Recent cell culture experiments sustain this hypothesis. Association of proteins to the plasma membrane can be mimicked by experimental inclusion of a myristoylation

site. Adding a myristoylation site to the N-terminus of Brk, enhanced its oncogenic role by promoting the proliferation, survival and migration of the human embryonic kidney cell line, HEK293. Trapping Brk in the nucleus with a synthetic NLS abrogated these effects (Kim and S.-T. Lee, 2009), indicating that Brk's oncogenic role may be dependent on its cellular localization. Brk's effects on β-catenin-mediated transcriptional activity were also found to be dependent on the cellular localization of Brk itself (Palka-Hamblin et al., 2010).

Without either a myristoylation site or a nuclear localization signal it is unclear how Brk delocalizes from one sub-cellular compartment to another and which cellular signals are responsible for controlling this transition. One plausible hypothesis is that it is the Brk-substrate interactions that regulate localization. We would therefore predict that Brk can 'travel' into the nucleus by 'piggy-backing' on a binding partner. The same hypothesis could be applied to cytoplasmic localization and indeed, supporting this theory, unpublished work from Angela Tyner's laboratory suggests that Brk is held in the cytoplasm by an as yet unidentified protein (Brauer and Tyner 2010). Although a less intriguing possibility from a research perspective, we should also not rule out that Brk may simply diffuse from one cellular location to another.

6. Brk interactions, substrates and activation

6.1 Substrates and interacting proteins

Brk is capable of phosphorylating a number of target molecules and a wealth of information on possible substrates and interacting proteins has been compiled. So far, at least 30 proteins have been shown to interact with Brk (summarised in Table 1), however, not all of these associations result in phosphorylation, neither do all of these proteins bind directly to Brk.

Many interactions are likely to be mediated via a 'third-party' that may be known, such as the signal transducing adapter protein-2 (STAP-2) which is also known as Brk kinase substrate (BKS), in the case of both signal transducing and activators of transcription STAT3 and STAT5 (Sekine et al., 2005; Ikeda et al., 2009; Ikeda et al., 2011) or by as yet unidentified interactions. Given that Brk is reported to have a kinase-independent function (Harvey and Crompton 2003), it is highly likely that not all the interacting proteins will be substrates of Brk's kinase activity. Brk may also function as an adaptor molecule; therefore one of Brk's functions could be to stabilize signalling complexes to allow phosphorylation of some of its interacting proteins (and/or additional molecules within the complex) by other kinases. Association of Brk in large signalling complexes, as an adaptor or scaffolding molecule, may also contribute to its cellular localization. The unidentified protein holding Brk in the cytoplasm that has been reported by Brauer and Tyner (Brauer and Tyner 2010) may shed further light on Brk's role as an adaptor protein.

Some of the proteins that interact with Brk have yet to be fully characterized. The protein which approximates to 100kDa and interacts with both Brk and BKS-STAP2 (Mitchell et al., 2000) has yet to be fully identified despite a number of known Brk substrates such as STAT5b, β-catenin and KAP3A being around 100kDa. Proteins such as β-catenin are not thought to be potential candidates (Mitchell et al., 2000) making it likely that the 100kDa protein will be identified as a *de novo* Brk-interacting protein.

The variety of binding partners identified indicates that Brk can interact and potentially regulate a number of significantly important pathways that are known to be involved in breast cancer cell growth and proliferation. For example, the importance of ErbB signalling in breast tumour progression has been well-documented (reviewed in Navolanic et al., 2003) and

clinical inhibition of this pathway with therapies such as trastuzumab and lapatinib is now routine for relevant sub-types of breast cancer (CRUK website). Brk associates with all 4 members of the ErbB receptor family (Aubele et al., 2008; 2010; Kamalati et al., 1996; 2000) and therefore is capable of promoting downstream signalling in response to ErbB ligand binding. Brk also attenuates EGFR signalling through interaction with and phosphorylation of ARAP1 (also known as Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology (PH) domain-containing protein 1) (Kang et al., 2010), as well as regulating possibly regulating signalling via reported interactions with PTEN and Akt (Aubele et al., 2008; Zhang et al., 2005).

Brk Substrates and Interacting Proteins	
Interacting Protein	**Localisation**
EGFR	Membrane
HER2	Membrane
ErbB3	Membrane
ErbB4	Membrane
IGF-1R	Membrane
*ARAP1	Membrane-associated
*Akt	Cytoplasmic/Membrane-associated
ADAM-15A	Membrane
ADAM-15B	Membrane
*β-Catenin	Membrane/Cytoplasmic/Nuclear
*KAP3A	Cytoplasmic/Nuclear
*STAT3	Cytoplasmic/Nuclear
*STAT5a/b	Cytoplasmic/Nuclear
IRS-1	Cytoplasmic/Membrane-associated
*IRS-4	Cytoplasmic/Membrane-associated
Erk5	Cytoplasmic
Erk	Cytoplasmic
MAPK	Cytoplasmic
PTEN	Cytoplasmic
*Paxillin	Cytoplasmic
*BKS-STAP-2	Cytoplasmic
*GNAS	Cytoplasmic
*FL139441	Cytoplasmic
GapA-p65	Cytoplasmic/Membrane-associated
*Sam68	Nuclear
*SLM-1	Nuclear
*SLM-2	Nuclear
PSF	Nuclear
*β-Tubulin	Cytoplasmic
*p190 Rho GAP	Cytoplasmic
23KDa	Cytoplasmic
100KDa	Cytoplasmic ?

Table 1. Brk Substrates and Interacting Proteins. Proteins known to interact with Brk are listed along with their usual cellular location. Proteins marked with * are confirmed as *bona fide* Brk substrates.

Research into insulin-like growth factor (IGF) signalling is also gaining momentum, especially given that IGF receptor (IGFR) expression is linked to poor outcome in ER-negative breast cancer patients (Railo et al., 1994) and IGF-1R expression and signalling are believed to mediate resistance to trastuzumab (Lu et al., 2001; Nahta et al., 2005). Immunoprecipitation and mass spectrometry experiments have identified insulin receptor substrate (IRS)-4 (IRS-4) as a Brk binding partner (Qiu et al., 2005). In HEK 293 cells, exogenous Brk and IRS-4 were demonstrated to associate in both resting and IGF1-1 stimulated cells. IGF-1 increased the phosphorylation of Brk in MCF-7 breast cancer cells, and this effect was enhanced in the presence of IRS-4 (Qui et al., 2005). Brk also co-precipitates with IRS-1 and IGF-1R in MCF10A-IGF1R cells (Irie et al., 2010).

Brk's association with ADAM-15 variants is of particular interest. Brk showed strong binding to ADAM-15A and ADAM-15B, but not ADAM-15C. Of the 4 alternatively spliced isoforms that were differentially expressed in human breast carcinoma tissue, high expression of ADAM-15A and ADAM-15B were associated with poor relapse-free survival in node-negative breast cancer patients, whereas higher levels of ADAM-15C appeared to predict a more favourable outcome (Zhong et al., 2008).

In addition to phosphorylating Sam68 and polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF), Brk can also bind to and phosphorylate the SLM-1 and SLM-2 RNA binding proteins (Haegebarth et al., 2004), as well as the transcription factors NFκB (Chakraborty et al., 2008), STAT3 (Liu et al., 2006) and STAT5b (Weaver and Silva 2007). Both Brk and its alternatively spliced isoform ALT-PTK6 have been reported to bind to β-Catenin (Palka-Hamblin et al., 2010; Brauer et al., 2011). Nuclear-targeted Brk negatively regulated β-Catenin/TCF transcription, whereas membrane associated Brk enhanced transcription (Palka-Hamblin et al., 2010); expression of ALT-PTK6, downregulated PTK6 activity and enhanced the inhibition of β-Catenin/TCF transcription that was mediated by Brk (Brauer et al., 2011).

Therefore Brk is capable of regulating both gene transcription and post-transcriptional RNA processing, although the outcome of this regulation will be dependent of Brk's cellular localisation.

6.2 Brk activation

As might be expected from the spectra of protein-protein interactions, Brk is activated by a number of different ligands (Figure 2), as well as exhibiting a small amount of basal autophosphorylation in *in vitro* kinase assays (Castro and Lange, 2010).

Unsurprisingly the signalling via ErbB and IGF-1R receptor ligands, EGF and IGF, activates Brk (Kamalati et al., 1996; Ostrander et al., 2007; Qiu et al., 2005). Activation of the MET receptor by hepatocyte growth factor (HGF) also activated Brk in both breast cancer and keratinocyte cell lines (Castro and Lange 2010). As all these growth factors have roles in cell proliferation, Brk activation by these ligands could increase the proliferative index of tumours. Activation of Brk by osteopontin (OPN) (Chakraborty et al., 2008), a chemokine-like protein that is known to enhance metastasis (Denhardt et al., 2003), has consequences for tumour progression if downstream signalling from Brk promotes the effects of OPN.

Keratinocyte differentiation is also important in terms of Brk-mediated biology. Brk can be activated in response to calcium or ionomycin, although in this context Sik/Brk activation results in the initiation of differentiation (Vasioukhin and Tyner 1997; T.C. Wang et al., 2005). Combined with the fact that Sik promotes differentiation of murine intestinal cells

(Haegebarth et al., 2006), the activation of Brk clearly has the potential for cell-type specific consequences that could be exploited for therapeutic purposes (see section 9).

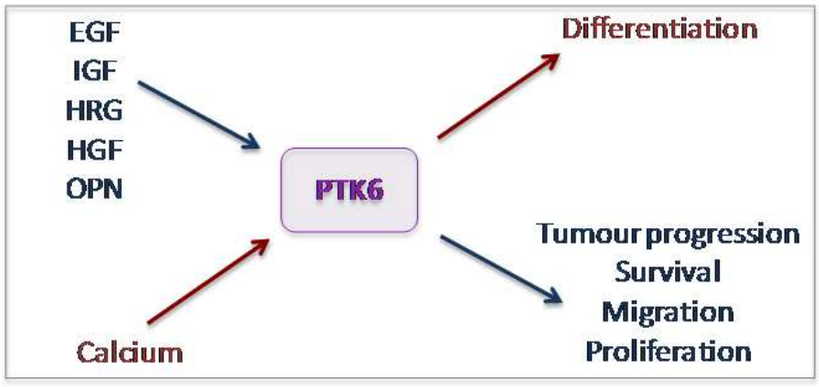

Fig. 2. Brk is activated by a number of ligands. Activation pathways that result in effects considered to be tumour promoting are shown in blue, and those resulting in differentiation are in brown.

6.3 Negative regulation of Brk

Little is known about the negative regulation of Brk activity, as much emphasis has been placed on trying to understand how Brk is activated and the biological effects of this activation. However, studies have demonstrated that the cytokine signalling suppressor, SOCS3, is able to inhibit Brk activity and the subsequent phosphorylation and transcriptional activity of STAT3 (Liu et al., 2006).

Sam68 has anti-proliferative properties and it has been suggested that these are neutralised, possibly by Brk, in breast cancer cells (Lukong et al., 2005). Over-expression of Sam68 in rat astrocytes has been shown to inhibit Brk-induced cell cycle progression (Lukong et al., 2005), indicating that the balance of expression, as well as localisation of both Brk and Sam68 could be important in mediating breast cancer cell proliferation.

7. Tumour-related effects of Brk expression

The elevated levels of Brk expression in tumour samples relative to the restricted levels in normal or differentiating tissues suggest that Brk may have a role in the processes underlying tumourigenesis, such as promotion of cancer cell proliferation and migration and evasion of cell death (Reviewed in Hanahan and Weinberg 2000; 2011).

7.1 Proliferation and cell cycle progression

There is a mounting body of evidence highlighting Brk's role in promoting proliferation and cell cycle progression in breast tumour cells. Brk can increase breast cancer cell proliferation as well as anchorage independent growth in normal mammary epithelial cells (Harvey and Crompton, 2003; Kamalati et al., 1996; Ostrander et al., 2007) and suppression of Brk levels by RNA interference has been shown to result in decreased proliferation in breast cancer cells (Chan and Nimnual, 2010; Harvey and Crompton, 2003; Ostrander et al., 2007).

Brk's role in promoting proliferation in response to EGF remains the best characterised. Brk has been shown to promote proliferation of the mammary epithelial cell line, Hb4a by

potentiating the effects of EGF, associating with EGFR and also recruiting PI3-K to ErbB3 receptors resulting in increased Akt activation (Kamalati et al., 1996; 2000). Furthermore, co-expression of HER2 and Brk in the non-tumourigenic breast cell line, MCF10A, was shown to increase the levels of Cyclin E and decrease p27 to induce cell cycle progression as well as increase Akt phosphorylation (Xiang et al., 2008). As Brk can directly activate Akt by phosphorylation of tyrosine residues 315 and 326 (Zheng et al., 2010), Brk could directly contribute to downstream signalling and presumably increased proliferation through interactions with Akt, as well as enhancing proliferation in response to EGF via activation of p190RhoGAP (Shen et al., 2008). EGF-mediated activation of Brk induced phosphorylation of Sam68 and promoted cell cycle progression, suggesting that Brk's oncogenic functions are, in part, mediated by inhibiting the tumour-suppressive functions of molecules such as Sam68 (Lukong et al., 2005; reviewed in Brauer and Tyner, 2010). The combined EGFR/HER2 inhibitor lapatinib inhibits HER2 mediated proliferation, however over-expression of Brk in MCF10A-HER2 cells reduced the effectiveness of lapatinib in inhibiting proliferation (Xiang et al., 2008).

Other ErbB receptor ligands are also capable of mediating receptor-Brk interactions and altering its activity. For example, heregulin has been shown to activate Brk's kinase activity in T-47D breast cancer cells (Ostrander et al., 2007). Increased proliferation as a result of activated Brk in response to stimulation with either EGF or heregulin resulted in activation of Rac GTPase, extracellular signal regulated kinase (ERK) 5, and p38 mitogen-activated protein kinase (MAPK), as well as an increase in Cyclin D1 expression (Ostrander et al., 2007).

Additional downstream targets of Brk that are involved in augmenting Brk's effects in promoting proliferation include STAT3 (Liu et al., 2006) and STAT5b (Weaver and Silva, 2007). Phosphorylation of both these proteins resulted in increased proliferation and transcriptional activity.

Brk's role in promoting proliferation is one way in which it can contribute to breast cancer development and progression. Interestingly however, Lukong and colleagues showed that Brk could phosphorylate the nuclear protein PSF causing it to delocalise to the cytoplasm resulting in growth arrest (Lukong et al., 2009). Their data provide mechanistic evidence supporting previous studies that cytoplasmic Brk is oncogenic, whereas nuclear Brk could play a role in negatively regulating cell cycle progression and/or proliferation (Derry et al., 2003; Kim and S.-T. Lee, 2009).

There is also some evidence to suggest that Brk does not always promote proliferation when transfected into non-transformed cells. Brk over-expression in rat fibroblasts did not promote either anchorage independent growth or cell cycle progression, but did affect cellular responses to DNA-damage and stress (Haegebarth et al., 2005). Intestinal epithelial cells from PTK null mice showed an increase in basal levels of proliferation compared to cells from wild-type mice, but in response to γ-irradiation both proliferation and BrdU labelling were increased in wild-type cells compared to PTK6 null cells (Haegebarth et al., 2009).

7.2 Cell death

Brk has been shown to participate in a number of signalling pathways that could ultimately regulate cell death depending on the cellular context. The disruption of the cell-cell matrix interactions acts a stimulus for apoptosis (Frisch and Francis, 1994), and given that Brk has been shown to transform mammary epithelial cells such that they proliferate in anchorage

independent conditions (Kamalati et al., 1996), it is likely that Brk also promotes anchorage-independent cell survival by protecting cells from cell death. Recent data has shown that Brk, via IGF-1 signalling, protected breast cancer cells from classical apoptosis/anoikis (Irie et al., 2010). In a separate study, Brk expression reduced breast cancer cell death via an autophagic pathway (Harvey et al., 2009). These studies indicate that PTK6 can protect cells from different types of programmed cell death.

The Bcl-x alternatively spliced variants, Bcl-x_L and Bcl-x_S, were shown to be differentially expressed in response to Brk targeting (Harvey et al., 2009); Brk suppression resulted in a concurrent reduction in the anti-apoptotic Bcl-x_L and an induction in Bcl-x_S, suggesting that targeting Brk could modulate a tumour cell's capacity for cell death. Investigations as to whether Brk mediates these effects in breast cancer cells through regulation of alternative splicing or through protein stability are on-going, however the Brk substrate Sam68 (Lukong et al., 2005) is able to regulate the alternative splicing of Bcl-x in HEK293 cells (Paranetto et al., 2007).

Contrary to the data outlined above, Brk sensitizes non-transformed rat fibroblasts to inducers of apoptosis such as serum starvation and UV irradiation/serum starvation (Haegebarth et al., 2005). As previously discussed (Harvey et al., 2009) parallels can be drawn with data on the c-*myc* oncogene, as Myc is also capable of sensitizing fibroblasts to induction of apoptosis by serum deprivation (Evan et al., 1992; Harrington et al 1994). In intestinal crypt epithelial cells PTK6 is induced by stress and promotes apoptosis through inhibition of Akt and Erk1/2 (Haegebarth et al., 2009). This further underlines the fact that that the pro-survival functions of Brk are likely to be dependent on cellular context.

7.3 Migration

Brk has been shown to promote the migration of breast cancer cell lines towards Heregulin, HGF and EGF (Castro and Lange 2010; H.-Y. Chen et al., 2004; Ostrander et al., 2007). Through its interaction with paxillin, Brk mediated EGF-induced migration and invasion of breast tumour cells has also been demonstrated to occur via a mechanism involving CrkII and Rac (Chen et al., 2004). As well as promoting proliferation, Brk phosphorylation of p190RhoGAP promoted migration and invasion (Shen et al., 2008), and KAP3A has been identified as physiological substrate of Brk during migration of BT20 breast cancer cells (Lukong and Richard 2008).

Studies to date therefore suggest that Brk can promote the migration of breast cancer cells through more than one mechanism, and in response to a number of different ligands.

7.4 Tumour formation

The *in vitro* data in a number of tumour types, but especially breast cancer, strongly support a role for Brk in augmenting some of the processes underlying breast cancer progression and dissemination.

Brk promoted ErbB2 induced tumourigensis in orthoptic transplantation-based models. Cells co-expressing Brk and ErbB2 formed tumours 2-3 weeks earlier, on average, than cells with ErbB2 alone. The ErbB2/Brk positive tumours also showed increased proliferation compared with ErbB2-only tumours (Xiang et al., 2008). OPN enhanced VEGF-dependent tumour progression in xenograft models, and in vitro experiments suggest that this is through activation of a PTK6/NFκB/ATF-4 signalling cascade (Chakraborty et al., 2008). There exists, in this scenario, potential for a feedback loop whereby Brk activation of NFκB

results in increased Brk expression, given that NFκB binding sites have been identified in the *ptk6* gene promoter (Kang et al., 2002; Mitchell et al., 1997).

7.5 Role of Brk's kinase domain in Brk-mediated biology

Brk has been reported to have kinase-independent function and it is proposed that it may act as an adaptor protein in signal transduction (Harvey and Crompton, 2003). Certainly the kinase inactive PTK6 K219M mutant is capable of promoting proliferation in over-expression studies in the PTK6 positive cell line T-47D, (Harvey and Crompton 2003) and Brk's association with, and regulation of, β-catenin is also independent of kinase activity (Palka-Hamblin et al., 2010).

However, certain aspects of Brk function including anchorage independent growth, and regulation of cell death phenotypes appear to require functional kinase activity (Harvey et al., 2009; Irie et al., 2010; Kamalati et al., 1996).

The reliance on functional kinase activity in migration is more clearly defined. Migration of breast cancer cells towards EGF and foetal bovine serum appeared to require Brk's kinase activity (H.-Y. Chen et al., 2004; Lukong and Richard 2008), however kinase inactive Brk was found to be able to promote migration towards HGF (Castro and Lange 2010).

Kinase inactive Brk did not appear to bind to ARAP-1 as well as wild-type Brk, indicating that the catalytic activity of Brk is required for the interaction with ARAP-1 and the maintenance of EGFR protein and the subsequent prolonging of EGFR signalling (Kang et al., 2010).

8. Brk and breast cancer patient prognosis

The literature relating to Brk expression and breast cancer prognosis is conflicted. Much of the *in vitro* cell culture data, support an oncogenic role for Brk as it has been shown to augment breast cancer cell proliferation and migration, ErbB receptor signalling, as well as inhibit cell death via different mechanisms. Gene expression data (Harvey et al., 2009) and immunohistochemistry staining (Aubele et al., 2007; Ostrander et al., 2007) from different cohorts of patient tumour samples indicated that Brk expression is correlated with higher-grade tumours initially suggesting that expression is likely to be linked with poorer prognosis for breast cancer patients as these tumours are more likely to disseminate (Porter et al., 2004). Indeed a recent study indicates that high Brk expression is associated with adverse patient outcomes (Irie et al., 2010).

Conversely, Aubele and colleagues also showed that whilst initially the probability of disease-free-survival was lower for patients with higher levels of Brk expression, beyond 50-100 months high Brk expression was linked with an improved probability of distant recurrence-free survival (P=0.001 at 240 months) (Aubele et al., 2007). As we have previously discussed one explanation for this discrepancy is that PTK6 expression may be correlated with expression of the oestrogen receptor, a known positive prognostic indicator (discussed in Harvey et al., 2009). The underlying cause of Brk overexpression may also contribute to patient outcome, especially in tumours where the *ptk6* gene is amplified as 20q and 20q13 amplifications have been associated with poor prognosis and more aggressive tumour phenotypes (Isola et al., 1995; Tanner et al., 1995).

In addition, it is also possible that driving tumour cells to proliferate may make them more susceptible to the effects of conventional chemotherapy agents. These are known to target actively-dividing rather than 'resting' cells, so increasing susceptibility to these agents

would enhance the benefits of such therapy thereby aiding patient survival. It would be particularly beneficial to examine patient survival in relation to both Brk expression and combination of therapy received before a definitive conclusion on the role of Brk expression in patient prognosis can be reached.

9. Brk-targeted therapies: Opportunities and implications

Brk has been considered as a potential therapeutic target for breast cancer for a number of years (reviewed in Harvey and Crompton 2004) and the wealth of more recently published *in vitro* and *in vivo* data, supports this hypothesis. Inhibition of Brk would be predicted to reduce breast cancer cell proliferation, migration and survival, as well as down-regulate some of the processes underlying tumour development and possibly dissemination. Given the high percentage of breast cancers that express Brk (up to 86%) (Harvey et al., 2009; Ostrander et al., 2007), a high proportion of patients could benefit from such a therapy.

The fact that the *ptk6*-null mouse survives into adulthood, is fertile, and there are minimal effects on health apart from the developmental issues such as changes in crypt length (Haegebarth et al., 2006), suggests that a Brk targeted therapy could be tolerable to patients. The biggest risk factor for developing breast cancer is increased age (BCC website), meaning that patients are diagnosed with breast cancer at a time in their life when development has already been completed, indicating that a Brk-targeted therapy would not cause any development-related adverse effects. In colonic crypt cells, Brk is induced in response to external stresses (Haegebarth et al., 2009) so any potential effects of Brk inhibitors on colonic cells may need to be monitored. PTK6 has been shown to sensitise cell to inducers of cell death in fibroblasts (Haegebarth et al., 2005), however this is unlikely to have any clinical impact as fibroblasts have yet to be shown as sites of PTK6 expression.

As well as inhibiting proliferation, survival and migration, Brk inhibition should reduce the signalling mediated via ErbB and IGF receptors. As inhibitors for both these receptor families are currently in clinical use or in clinical trials as combination therapies, it could be hoped that therapeutic inhibition of Brk could produce similar anti-tumour effects. Co-targeting Brk alongside EGFR or Her-2, may produce significant clinical benefit especially in tumours where both ptk6 and ErbB2 are over-expressed or co-amplified. Brk has already been implicated in mediating resistance in *in vitro* studies to the dual EGFR/Her-2 inhibitor, lapatinib (Xiang et al., 2008), suggesting that co-targeting of ErbB receptors and Brk is of therapeutic value.

Brk-targeted therapies may well have consequences for expression of cell death related proteins, as Brk suppression has been shown to modulate Bcl-x_L:Bcl-x_S ratios in favour of Bcl-x_S (Harvey et al., 2009). The induction of Bcl-x_L has been linked with resistance to both traditional chemotherapy agents as well anti-hormonal treatments in breast cancer (Kumar et al., 1996; Mercatante et al., 2002; Minn et al., 1995). As reducing Bcl-x_L or over-expressing Bcl-x_S increases the sensitivity of response to chemotherapeutic agents in breast cancer cell lines (Simões-Wüst et al., 2002; Sumantran et al., 1995), targeting Brk may also modulate chemotherapeutic responses to existing treatments. This possibility is of particular importance in triple negative breast cancers that are intrinsically less sensitive to chemotherapeutic agents and where, due to their negative-receptor status, targeted therapies are not suitable.

If combinations of already existing therapies, such as radiation, are to be used alongside Brk inhibitors, treatment protocols/rationales will need to be strictly determined. Brk is induced

with DNA-damage in response to γ-irradiation (Haegebarth et al., 2009), meaning that the order of treatment for patients will need careful consideration.

Kinase inhibitors for a number of cellular targets such as the kinase domains of EGFR and HER2, and bcr-abl are now widely used, and the side effects appear to be manageable and the drugs are tolerated reasonably well by patients. A kinase inhibition strategy is often the preferred option by pharmaceutical companies (reviewed in Keri et al., 2006) and one that could be considered for Brk. This strategy is not without issues as Brk is reported to have kinase independent role in both proliferation (Harvey and Crompton, 2003) and the regulation of β-catenin (Palka-Hamblin et al., 2010) suggesting that perhaps targeting of the SH2 or SH3 domains maybe more beneficial (Harvey and Crompton, 2004). However, there is an increasing body of knowledge showing that anchorage independent growth and cell death (Harvey et al., 2009; Irie et al., 2010; Kamalati et al., 1996), as well as migration (H.-Y. Chen et al., 2004; Lukong and Richard, 2008) and regulation of EGFR signalling (Kang et al., 2010) do require kinase function suggesting, that while caution is required, inhibition of kinase activity would offer some clinical benefit via inhibition of these processes. Data presented in 2010 at the American Association for Cancer Research annual conference suggest that kinase inhibitors are being considered by the pharmaceutical industry as Brk-targeted therapies (Y. Wang et al., 2011).

We have previously discussed the implication for kinase inhibition and the potential effects on differentiation (Harvey and Crompton 2004), however given that EGF signalling is heavily implicated in keratinocyte differentiation (Nanney et al., 1990; Peus et al., 1997) and that EGFR and Brk are proposed to be co-regulated in differentiation (Tupper et al., 2011), it is possible that the adverse effects would be no greater than those observed with EGFR inhibitors.

10. Future perspectives

Inhibiting Brk remains an attractive option for the treatment of breast cancer patients. However, there are a number of 'knowledge gaps' that need to be addressed.

The role of ALT-PTK6 in regulating PTK6 function could be vital to furthering our understanding of how Brk is negatively regulated. If SH3 inhibition proves to be an appropriate therapeutic strategy, understanding ALT-PTK6 function, as an SH3-only protein, will be important.

There are a number of groups working within the PTK6 research community and some of our knowledge of PTK6 function is gained from studies on other tumour types such as prostate and oral squamous carcinomas (Derry et al., 2003; Petro et al., 2004). There is now a need to assess whether the same conclusions can be made for breast cancer.

PTK6 expression is increased with increasing tumour grade in breast cancer, however the clinical implications of Brk expression need further clarification. Is PTK6 a negative prognostic indicator? Or does patient outcome depend on treatment regime in the context of Brk expression?

One of the major 'knowledge gaps' is our lack of understanding as to how or why *ptk6* gene expression is triggered in breast cancer cells, when expression is absent in normal mammary development. What are the underlying events that 'switch-on' expression?

Many Brk-protein associations and interactions have been shown a wide range of cell types, some by over expression studies of both Brk and its proposed substrate/binding partner. Further studies are now warranted to determine which of these reported interactions are relevant from a pathological perspective. These investigations, combined with fully understanding Brk's cellular localisation and how it translocates from one compartment to

another, will be particularly helpful especially in the context of designing SH3 or SH2 inhibitors to disrupt specific disease-related protein-protein interactions.

11. Conclusion

The relatively limited physiological expression profile of Brk, and its high level of *de novo* expression in breast tumours make it an attractive therapeutic target. Much progress has been made in the last decade in our understanding of Brk's role in the processes underlying tumour development (summarised in Figure 3). More recent studies indicate that a kinase-inhibitor approach to 'anti-Brk' drug development may warrant further investigation. The next decade will undoubtedly be crucial for providing further knowledge that will consolidate Brk as a viable therapeutic target for breast cancer.

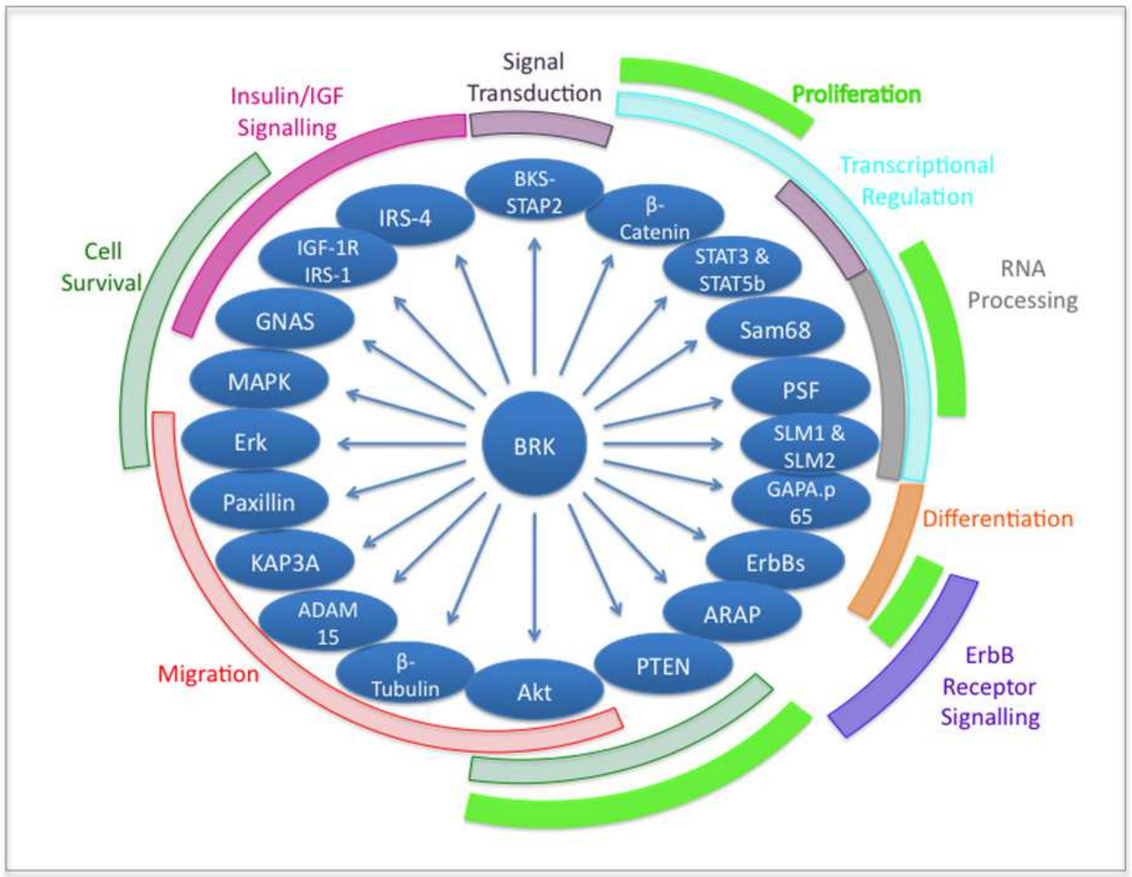

Fig. 3. Summary of the known interactions of Brk and the proposed biological effects that are regulated by these interactions.

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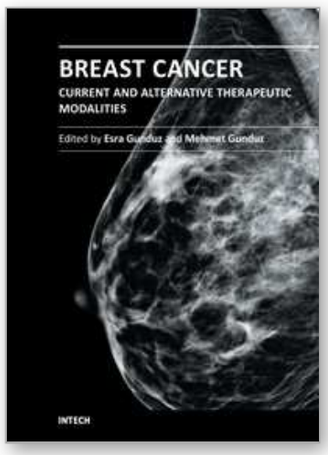

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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